Egyptian Journal of Aquatic Research (2013) 39, 185–192

National Institute of Oceanography and Fisheries

Egyptian Journal of Aquatic Research

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FULL LENGTH ARTICLE

Microcystin bioaccumulation can cause potential mutagenic effects in farm fish

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Received 26 August 2013; revised 1 November 2013; accepted 1 November 2013
Available online 5 December 2013

KEYWORDS

Cyanobacterial blooms;
Cyanotoxins;
Semi-arid region;
Fish farm;
Oreochromis niloticus

Abstract This study investigated the bioaccumulation of microcystin and its potential mutagenic effects in cage-reared fish. For six months, three reservoirs (Acauã, Cordeiro and Camalau) in arid regions of Brazil were monitored. After five months, 10 fish (*Oreochromis niloticus*) were collected from each tank, and the amounts of microcystin in their muscles and viscera were analysed. Mutagenic effects were also evaluated. Species of cyanobacteria were present during all months of our study. The highest biovolume of cyanobacteria and microcystin in water occurred in the Acauã Reservoir; this was followed by Cordeiro and Camalau. All fish sampled contained microcystin, ranging from 16.01 to 37.09 ng g^{-1} in muscle and 228.2–804.0 μg g^{-1} in liver. We observed significant differences between the micronucleus count in experimental and control fish ($p < 0.05$), suggesting a potential mutagenetic effect in the experimental fish. In all reservoirs, the fish microcystin level was well above the World Health Organization (WHO) tolerable daily intake, indicating a serious risk to consumers.

Introduction

Concern regarding the effects of cyanobacteria on human health has been raised in many countries (Schwimmer and Schwimmer, 1968; Carmichael et al., 1988; Sivonen, 1996; Baker and Humpage, 1994; Bouvy et al., 2003). Cases of cyanobacterial blooms in reservoirs and lakes are a matter of public health. Intense cyanobacteria blooms are prolific in

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Peer review under responsibility of National Institute of Oceanography and Fisheries.

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http://dx.doi.org/10.1016/j.ejar.2013.11.002

eutrophic conditions (increased nitrogen and phosphorus concentrations), and these commonly result from anthropogenic pollution (Granéli et al., 2008; Heisler et al., 2008). Cyanobacterial blooms affect water quality by changing pH, transparency and biodiversity, and by producing odours and/or toxins such as cyanotoxins (Blahová et al., 2008). Microcystins (MCYST) are cyclic heptapeptides produced mainly by *Microcystis* sp. and are regarded as the most common and one of the most dangerous groups of cyanobacterial toxins (Zhang et al., 2009).

Exposure to cyanotoxins can occur through different routes, including dermal, respiratory and oral. Fish can be exposed to MCYST either actively, during feeding, or passively, when the toxins pass through the gills during breathing (Malbrouck and Kestemont, 2006). Fish at the top of the aquatic food chain are likely to be the most profoundly affected by exposure to MCYST; consumption of these fish may pose a great risk to humans (Magalhães et al., 2001).

In farm-raised fish under conditions of confinement, or during extended blooms of toxic cyanobacteria in reservoirs, a decrease in predation may not be sufficient to protect these species and other planktivorous fish from acute intoxication. Thus, farm fishing, an important source of protein for the world population, may be compromised by cyanobacterial blooms.

To protect consumers from the adverse effects of cyanobacterial toxins, the World Health Organization (WHO) established the maximum allowable concentration for MYCST in drinking water as $1\ \mu g\ L^{-1}$ and a tolerable daily intake (TDI) of 0.04 μg/kg of body weight per day as a provisional guideline (Chorus and Bartram, 1999). It was assumed that 80% of the MCYST ingested on a daily basis came from contaminated drinking water and that the remaining 20% was taken in via food or inhalation (Dietrich and Hoeger, 2005).

Fish are excellent subjects for the study of the mutagenic and/or carcinogenic potential of contaminants present in water samples because they can metabolise, concentrate and store waterborne pollutants (Ali et al., 2008). Monitoring both drinking water supplies and fish exposed to these toxins is of critical importance to appropriately assess the resultant environmental and health risks.

However, very few field studies have investigated the bioaccumulation of MCYST in aquatic organisms, therefore information on MCYST content in shrimps, prawns, crayfish and fish collected from natural freshwater environments is very scarce (Chen et al., 2005, 2007; Deblois et al., 2011). There are a few studies examining MCYST content in the freshwater species *Cyprinus carpio* (Fischer and Dietrich, 2000), *Tilapia rendalli* (Magalhães et al., 2001), *Oreochromis niloticus* (Mohamed et al., 2003; Deblois et al., 2011), some phytoplanktivorous fish (silvercarp, *Hypophthalmichthys molitrix*) (Xie et al., 2005; Chen et al., 2007) and omnivorous fish such as *Carassius gibelio* (Kagalou et al., 2008). The mutagenic effects of MCYST in fish were reported by Al-Sabti and Metcalfe (1995), Bolognesi et al. (1999), Grisolia and Starling (2001), Prieto et al. (2006) and Moreno et al. (2009).

The purpose of the present study was to report MCYST levels in seston of waters used in farm fishing and assess potential bioaccumulation of MCYST in fish (*O. niloticus*). Additionally, the potential mutagenic effects induced by exposure to and bioaccumulation of MCYST in fish to discussed.

Material and methods

Description of the study area

The study was conducted in three reservoirs located in the Paraiba River Basin in a semi-arid region of Brazil. These reservoirs were selected for survey of cyanobacteria and presence of fish farms. The reservoirs are used to supply water to the local population. In the last few years, fish farming has grown into an intensive and highly profitable form of aquaculture in this area, especially with regard to *O. niloticus* culture.

The Acauã Reservoir (7°27.5′3″S, 35°35′52.6″W) occupies an area of 17.8 km^2, with a mean depth of 25 m, and an accumulation capacity of approximately 253×10^6 m^3. The Cordeiro reservoir (7°47′38.00″S, 36°40′14.04″W) has a capacity of approximately 70×10^6 m^3, covers 11.23 km^2 and has a mean depth of 8.3 m. The Camalau reservoir (7°53′33.94″S, 36°50′39.16″W) covers 8 km^2, has a capacity of approximately 47×10^6 m^3 and a mean depth of 12.5 m (Fig. 1). All reservoirs are polymictic and eutrophic.

Water sample collection

Bimonthly samples were performed during a period of five months between August and December of 2009. Environmental and phytoplankton samples were collected by a Van Dorn Bottle held 10–20 cm beneath the water surface at one permanent site next to the fish farming region of the reservoirs. Environmental variables evaluated were: water temperature (digital thermometer with 0.1 °C accuracy), pH (portable membrane pH meter), dissolved oxygen (digital oximeter) and water transparency (Secchi disk). Water samples were collected in PVC bottles previously cleaned with distilled water and stored in receptacles containing ice for transportation to the lab, where they were frozen and nutrients (inorganic dissolved nitrogen and total phosphorous) were examined according to the procedures described by APHA (1992). Chlorophyll *a* concentrations were determined spectrophotometrically based on the procedures described in APHA (1995). Sub-samples (300 ml) were preserved in Lugol's solution and observed under an inverted microscope using five 25-ml sedimentation chambers for cyanobacteria identification and quantification (Utermöhl, 1958). Cyanobacterial genera and species were identified via microscopic observation by distinguishing morphological characteristics cited in the literature (Komarek and Agnostidis, 1986; Baker, 1991, 1992). Cyanobacteria biovolume was obtained by multiplication of population density by individual volume (Hillebrand et al., 1999). Spearman correlation analysis was used to demonstrate relationships between cyanobacteria biovolume and physical and chemical variables. These statistical tests were performed using the statistical software Statistica 7.

MCYST determination in natural waters

The determination of MCYST in natural water samples was performed using a commercially-available ELISA Microcystin Plate Kit (ENVIROLOGIX INC.®). This assay uses antibodies against microcystin-LR. MCYST was extracted from 20 to 100 mg of lyophilised seston fractions in 10 ml of 0.1% (v:v)

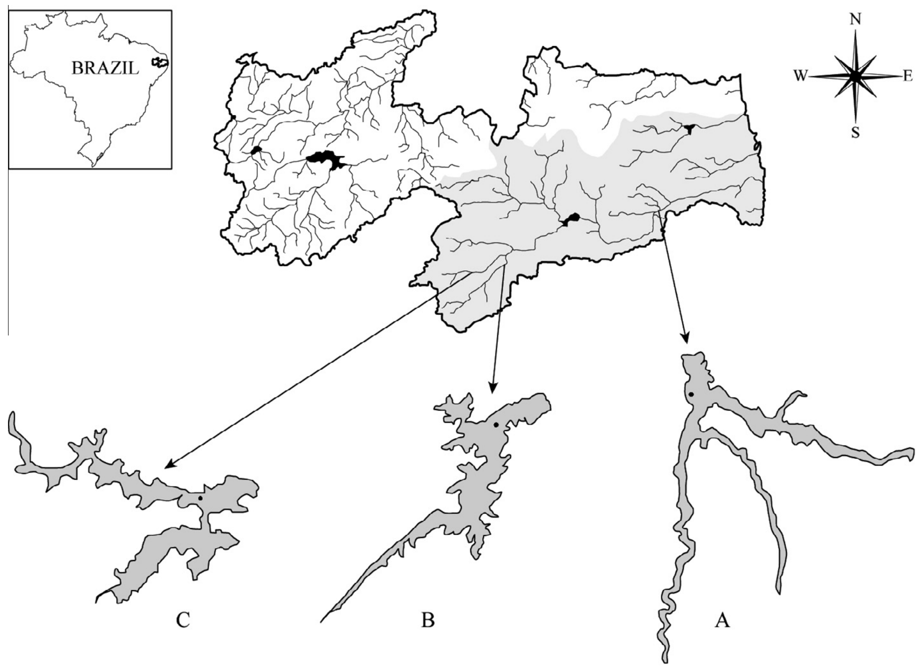

Figure 1 Localisation of sampling points: (A) Acauã Reservoir, (B) Cordeiro Reservoir and (C) Camalau Reservoir. Fish sampling points are indicated as "•" in the map.

trifluoroacetic acid (TFA) in methanol and agitated for one hour. The sample was then centrifuged at 11,000 rpm and the supernatant was collected and stored in a beaker. The pellet was re-extracted twice and the supernatants were pooled together at a final volume of 30 ml. The extract was completely evaporated and then resuspended in Milli-Q water. The resuspended sample was passed through a C18 cartridge which was washed and eluted with Milli-Q water, 20% methanol, 30% methanol and then with 0.1% TFA in methanol. This last fraction was evaporated and resuspended at the appropriate concentration for determination of microcystin.

MCYST bioaccumulation in fish (O. niloticus)

To examine the bioaccumulation of MCYST, fish were collected after five months of environmental exposure (December, 2009). Ten fish were purchased at fish farms located in the reservoirs of Acauã, Cordeiro and Camalau. Fish were sacrificed and the liver tissue and muscle were excised, weighed and immediately frozen at −20 °C at a field research station. In the laboratory, collected livers were frozen at −88 °C prior to MCYST analysis. Mean weight of the fishes in the Acauã Reservoir was 470 g (±28.3) with an average length of 25.6 cm (±1.14). For the fishes in the Cordeiro Reservoir, the mean weight was 670 g (±50.2) and mean length was 30.1 cm (±2.4). The fish from the Camalau Reservoir had a mean weight of approximately 518.18 g (±87.3) and a mean length of 28.8 cm (±2.2).

Fresh viscera and muscle samples (20 g) were minced and homogenised in 20 ml of 0.1% (v:v) TFA methanol. The sample was then centrifuged at 11,000 rpm and the supernatant collected and stored in a beaker. The pellet was re-extracted twice and the supernatants were pooled together at a final volume of 30 ml. The extracts were vacuum-filtered through fibreglass filters. The filtrates were completely evaporated and then resuspended in Milli-Q water. The filtrates were then passed through a C18 cartridge. This cartridge was washed and eluted with Milli-Q water, 20% methanol, 30% methanol and then with 0.1% TFA methanol. This last fraction was evaporated and resuspended at the appropriate concentration for the determination of microcystin. Muscle and viscera samples were analysed via immunoassay using the ELISA Microcystin Plate Kit (ENVIROLOGIX INC.®).

Micronucleus test

From the fish collected for analysis of MCYST bioaccumulation, aliquots were reserved for a peripheral blood micronucleus test. Blood samples were collected using heparinised syringes by gill venipuncture, and a drop of this blood was subsequently placed on one end of a clean, dry glass slide, and with the aid of a cover slip leaning at an angle of 45°, the blood was spread evenly, forming a thin layer. Two slides were prepared for each animal. After drying for 24 h, the slides were fixed in absolute methanol for 10 min, then stained with 10% Giemsa diluted in phosphate buffer pH 6.8 for 25 min and finally washed with distilled water. The slides were examined via optical microscopy with 1000× magnification, and each blood SQ made a count of 2000 erythrocytes.

For the control groups, we used ten specimens of *O. niloticus* from a reservoir with no recorded blooms of cyanobacteria. The positive control was created via a 30-h body exposure to cyclophosphamide at a concentration of 5 mg/L. The negative control group was also composed of 10 specimens of *O. niloticus*, and, for both groups, we prepared two slides per animal.

After cytological analysis of the blood samples, calculations were made of mean frequencies of micronucleated cells as well as the standard deviations for each treatment group. We applied the Mann–Whitney test to these results, performing comparisons between values obtained for each group of tanks and those obtained from the positive and negative control groups. Statistical tests were performed using the statistical software Statistica 7.

Results and discussion

Physical and chemical variables

The main physical and chemical characteristics of the reservoirs during the period of study are summarised in Table 1. All reservoirs were mixed, and the temperatures remained at approximately 28 °C at all sites. The dissolved-oxygen concentrations indicated the existence of oxygenated water columns. Water pH levels were alkaline to neutral, showing little variation throughout the reservoirs. Light penetration was low in water from Acauã and Cordeiro and moderate in water from Camalau. In the Acauã Reservoir, the average concentration of total phosphorus (TP) was 213.07 $\mu g\ L^{-1}$ ($\pm$91.65), in the Cordeiro Reservoir, the average concentration of TP was 395.1 $\mu g\ L^{-1}$ ($\pm$190) and in the Camalau Reservoir it was 49.07 $\mu g\ L^{-1}$ ($\pm$29.15). The average concentrations of dissolved inorganic nitrogen (DIN) were 126.4 $\mu g\ L^{-1}$ ($\pm$50.67), 203.68 $\mu g\ L^{-1}$ ($\pm$163.05) and 251.1 $\mu g\ L^{-1}$ ($\pm$131.4) in the Acauã, Cordeiro and Camalau reservoirs, respectively. The highest chlorophyll average was in the Acauã Reservoir, at 34.40 $\mu g\ L^{-1}$ ($\pm$6.17). The concentration in the Cordeiro Reservoir, 6.92 $\mu g\ L^{-1}$ ($\pm$10.49), followed, and the Camalau Reservoir had the lowest concentration, at 1.31 $\mu g\ L^{-1}$ ($\pm$0.28).

Diversity of cyanobacteria

The phytoplankton communities of the three reservoirs were represented by a total of 94 taxa (genus and species) from the following divisions: Chlorophyta, Cyanophyta, Bacilariophyta, Euglenophyta, Zygnemaphyceae, Oeodogonophyceae and Chlamydophyceae (Fig. 2). The Cyanophyta group was represented by 20 taxa at the Acauã and Cordeiro Reservoirs and 19 taxa at the Camalau Reservoir. The species *Cylindrospermopsis raciborskii* Woloszynska, *Microcystis aeroginosa* Kützing and *Planktothrix agardhii* Gomont were common in all reservoirs. *Sphaerocavum brasiliense* Azevedo and Sant'Anna occurred only in the Cordeiro Reservoir, and *Anabaena circinalis* Rabenhorst occurred only in the Acauã Reservoir.

With regard to the relative abundance of cyanobacteria, the highest biovolume occurred in the Acauã Reservoir at 38.7 $mm^3\ L^{-1}$($\pm$3.3), followed by the Cordeiro Reservoir at 18.4 $mm^3\ L^{-1}$ ($\pm$2.4) and the Camalau Reservoir at 2.6 $mm^3\ L^{-1}$ ($\pm$0.7). Temporal analyses revealed great changes in cyanobacterial species dominance in the reservoirs (Fig. 2). In the Cordeiro Reservoir, *Cilindrospermopsis raciborskii* was dominant in August (50.3% the total biovolume) and October, 2008 (51.2% of total biovolume), whereas *Microcystis protocystis* was dominant in December, 2008 (57.65% of total biovolume). In the Camalau Reservoir, *Plankthothrix agardhii* was dominant in August (54.1% of total biovolume) and December, 2008 (49.8% of total biovolume), whereas *M. protocystis* was dominant in October, 2008 (51.7% of total biovolume). In the Acauã reservoir, *P. agardhii* was dominant in all periods. The dominance of *P. agardhii* was associated with low concentration of DIN (−0.38; $p < 0.05$) and low water transparency (−0.31; $p < 0.05$), *M. protocystis* was associated with high concentrations of TP (0.37; $p < 0.05$) and *C. raciborskii* was associated with low water transparency (−0.34; $p < 0.05$), low DIN concentration (−0.57; $p < 0.05$) and high TP concentration (0.45; $p < 0.05$). All dominant species observed are potential producers of microcystins (MCYST).

Fish farmed in net cages is an important factor related to growth in nutrient concentrations, chlorophyll *a* and a reduction of water transparency. This activity is an important source of anthropogenic impacts on reservoirs (Starling et al., 2002; Lazzaro et al., 2003; Guo et al., 2009; Borges et al., 2010), observable simply by the release of dissolved or suspended metabolites derived from food. These substances are associated with high temperatures favouring an increase in the density of phytoplankton, particularly cyanobacteria (Padisák and Reynolds, 1998; Chorus and Bartram, 1999; Reynolds et al., 2002), which explained the high biovolume of cyanobacteria observed in all the reservoirs studied.

Some studies have shown that cyanobacteria may have adverse effects on fish, including damage to the liver, gills and kidneys, ionic disturbances, behavioural changes, reduced growth and mortality (Ericksson et al., 1989; Tencalla et al., 1994; Rodger et al., 1994; Li et al., 2005). Although large-scale fish mortality may be related to blooms of toxic algae and cyanobacteria (Ericksson et al., 1989; Rodger et al., 1994; Landsberg, 2002), other factors such as pH (due to photosynthetic activity and low oxygen concentration) associated with the decay of the bloom may also be linked to mortality (Christoffersen, 1996). However, one of the most damaging consequences of cyanobacteria blooms is the incorporation of cyanotoxins at different trophic levels (plankton, fish and man) (Magalhães et al., 2001).

Toxigenic cyanobacteria

MCYST was detected in the waters of all three reservoirs in all months during which samples were collected (Fig. 2). The

Table 1 Physical and chemical variables measured in reservoirs.

Variables	Average ± SE Acauã		Average ± SE Cordeiro		Average ± SE Camalau	
Temperature	26.33	±1.42	28.00	±0.78	28.17	±0.62
pH	8.37	±0.21	8.20	±0.16	7.69	±0.24
Water transparency	0.63	±0.05	0.80	±0.06	1.15	±0.33
Dissolved oxygen	9.83	±2.69	6.87	±1.63	5.97	±0.9
Dissolved inorganic nitrogen (DIN)	126.40	±50.67	203.67	±163.05	251.10	±131.43
Total phosphorus (TP)	213.07	±91.65	391.00	±190.69	49.07	±29.15
Chlorophyll *a*	34.40	±6.17	6.92	±0.49	1.31	±0.28

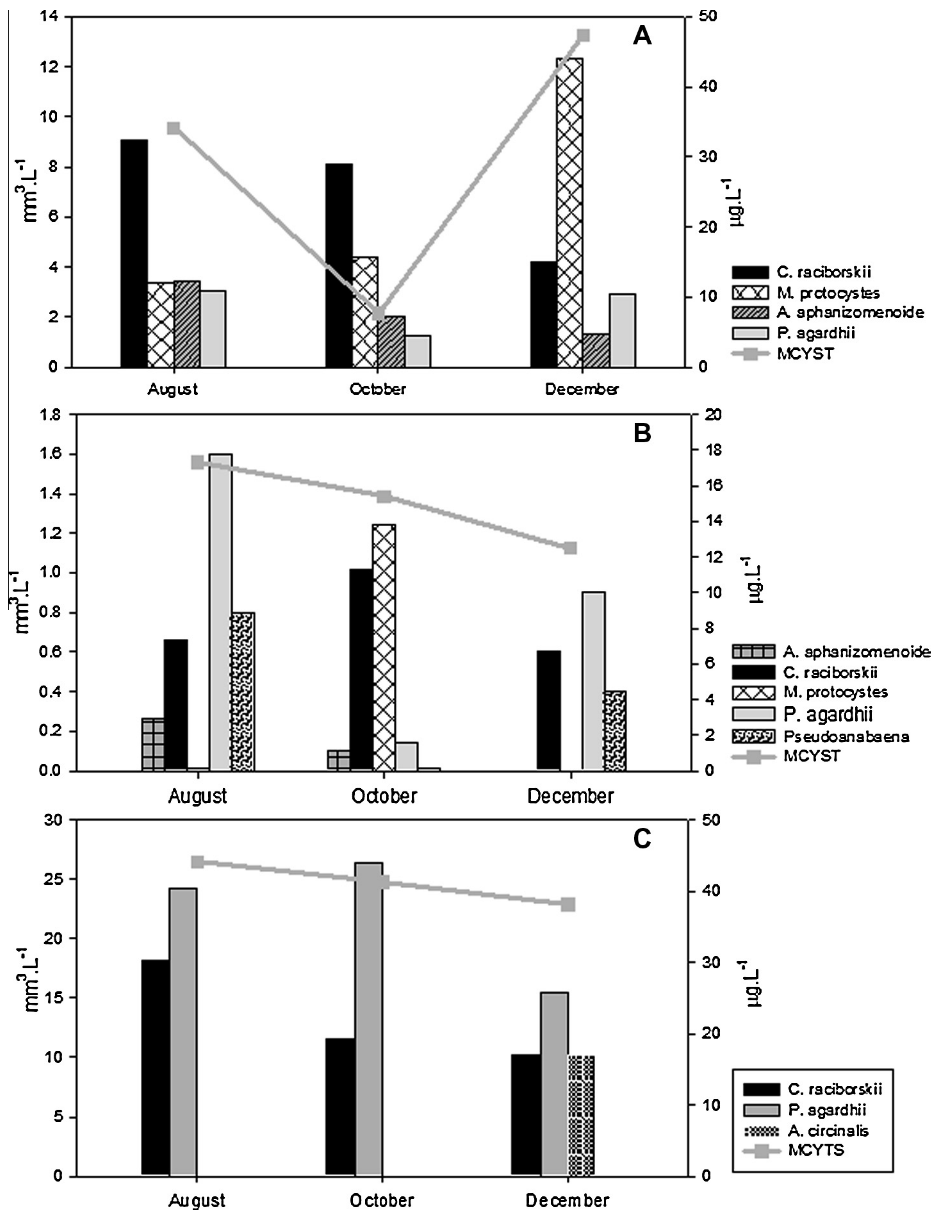

Figure 2 Changes in cyanobacterial biovolume and MCYST concentration in the Acauã (A), Cordeiro (B) and Camalau Reservoirs (C).

highest concentrations were observed in Acauã (41.16 µg L^{-1} ± 3.2), followed by Cordeiro (29.7 µg L^{-1} ± 20.1) and finally Camalau (15.0 µg L^{-1} ± 2.4). MCYST concentrations were associated with *M. protocystis* ($r = 0.41$; $p < 0.05$) and *P. agardhii* ($r = 0.49$; $p < 0.05$).

MCYST were detected in the muscle and viscera of all fish sampled. In the muscle, concentrations varied from 16.01 ng g^{-1} at the Camalau Reservoir to 37.09 ng g^{-1} at the Acauã Reservoir (Fig. 3A). In the viscera it varied from 228.2 µg g^{-1} at the Camalau reservoir to 804.0 µg g^{-1} at the Acauã Reservoir (Fig. 3B). Hence, the highest value of MCYST in fish muscle and viscera was found at the Acauã Reservoir.

The results of the micronucleus test are summarised in Table 2. Significant variations are observed in the averages of fish micronucleus cells between reservoirs. Clastogenetic effects represented by the formation of a micronucleus presented more values on Acauã followed by Camalau and Cordeiro Reservoir. We observed significant differences between the micronucleus count in experimental and control fish ($p < 0.05$), suggesting a potential mutagenetic effect in the experimental fish. Camalau reservoir measurements were similar to those from the Acauã Reservoir. In the Cordeiro Reservoir the difference between the micronuclei in experimental and control fish was insignificant (Table 2).

Tilapias's resistance to acute toxicity is not known. They may have a detoxification system that is able to metabolise the toxin to a less toxic compound (Wiegand et al., 1999). However, at a concentration of 800 mg kg^{-1} (maximum concentration in fish viscera), there should be enough MCYST to cause severe damage to fish (Tencalla et al., 1994; Magalhães et al., 2001; Soares et al., 2004). In this study, toxin exposure caused apparent mutagenetic effects in fish.

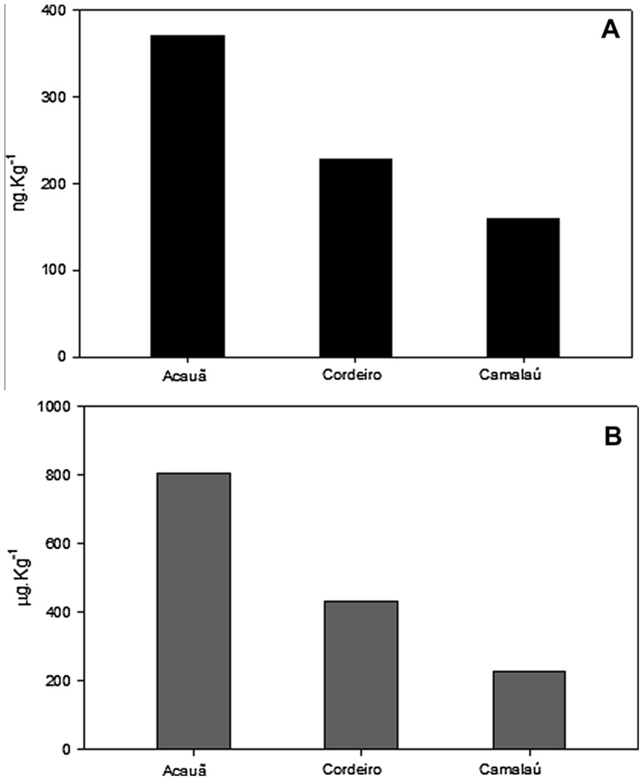

Figure 3 Microcystin concentration in the fish muscle (A) and fish viscera (B), from fish in the Acauã, Cordeiro and Camalau Reservoirs.

Table 2 Averages of micronucleus (MN) occurrence in fish.

Reservoir	MN	U-test
Negative control	1.05 ± 0.6	–
Acauã	3.65 ± 1.08	0.001*
Cordeiro	1.35 ± 0.7	0.244
Camalau	1.65 ± 0.8	0.026*

* Significant values ($p < 0.05$).

Tilapia can diminish their grazing rate when feeding on toxic cyanobacterial cells (Keshavanath et al., 1994). However, in the present study, MCYST accumulated in the viscera and muscle of all fish sampled. Evidently, faced with chronic exposure, tilapia cannot avoid the ingestion of toxins which can result in mycrocystin accumulation in fish tissues (Magalhães et al., 2001; Zhao et al., 2006; Deblois et al., 2008). Our results showed a rapid transference of MCYST from seston to fish. MCYST is thought to be taken up by a bile acid transporter in the intestinal and liver cells, with part of the toxin excreted via faeces and another part that accumulates in the liver and muscle tissue (Zhao et al., 2006; Magalhães et al., 2001).

MCYST content in tilapia muscle observed in the present study was quite high. If a human weighing 60 kg ingested 300 g of the muscle, the ingestion of MCYST would be 0.8–1.85 mg.kg^{-1} body weight/d. The tolerable daily intake (TDI) of MCYST as suggested by the WHO is 0.04 mg.kg^{-1} body weight/d (Chorus and Bartram, 1999). Our data confirmed the accumulation and persistence of MCYST in *O. niloticus* muscle and demonstrates a risk incurred upon consumption of this fish. All of the muscle samples examined were above the recommended TDI.

Our study demonstrated that MCYST accumulated in tissues has a potential to promote mutagenic effects in fish, and although not toxic to humans, may lead to levels consumed that are above the recommended levels of drinking water MCYST by the WHO. This scenario is grave, because case of deaths associated with cyanobacteria toxicity has occurred. These results suggest that a complex biological pattern of accumulation and depuration occurs in the fish, and that fish muscle can become toxic even after the termination of a toxic bloom. Regular monitoring of the concentration of MCYST in water and fish muscle is extremely important in farm fishing, and must be undertaken in order to evaluate the effects of chronic MCYST exposure in fish and guarantee food safety.

Acknowledgments

The authors are grateful to the Universidade Estadual da Paraíba, for logistical support, to researchers of the Laboratório de Ecofisiologia e Toxicologia de Cianobactérias at Universidade Federal do Rio de Janeiro for assistance in determining MCYST concentrations, to CNPq for financial support, to CAPES for a scholarship granted for the first author.

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